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ABSTRACT

Our proposal is focused on developing a versatile and generic technology platform that allows direct evaluation of the roles played by specific genes (and their products) in the genesis, progression and maintenance of breast cancer. We will construct genetically modified mice in which individual genes have been modified so as to incorporate a reversible switch. This will allow us to switch off (and on) individual target gene and determine what role that gene plays in mammary carcinoma. In effect, we can model the efficacy, specificity and side-effects of a drug designed to target that gene's function, either alone or in combination with existing therapies.

We will test our proposed technology platform by investigating the roles of two pervasive proto-oncogenes, c-*myc* and *E2f3*, in breast oncogenesis. Both c-*myc* and *E2f3* are pivotal determinants of mammary epithelial cell proliferation, differentiation, survival and tumor progression and excellent theoretical targets for breast cancer therapy. By blocking the functions of each of these genes, separately and together, we will for the first time evaluate their separate and combined roles in driving breast cancer progression, in the maintenance of established tumors, and as therapeutic targets.

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Introduction

Identifying the optimal molecular targets for effective and specific treatment of breast carcinoma is hampered by our woefully incomplete knowledge of the molecular lesions that underlie breast cancer initiation, progression and maintenance. Although tumors appear genetically complex, it seems likely that they are driven by a very restricted repertoire of molecular lesions that are required for their maintenance. To test this hypothesis, we proposed to construct a novel type of mouse cancer model in which endogenous genes encoding critical oncogenes are modified so that there expression can be modulated at will by the action of ligand-dependent heterologous repressors. Using such heterologous repressor targeting (HRT), we can directly assay the requirement for normal or deregulated expression of specific genes in the genesis, progression and maintenance of breast and other cancers induced by any means, including genetic manipulation, radiation or carcinogen exposure. Our initial focus within this BCRP proposal is on c-*myc* and *e2f3* genes, both of which encode pleiotropic transcription factors whose deregulated activity is causally implicated in breast (and other) cancers.

Our proposal has three principal aims, each divided into a number of discrete tasks. The principal aims are:

1. Construct genetically modified mice in which the endogenous c-*myc* and *e2f3* genes are rendered susceptible to ectopic control by either the IPTG-dependent Lac or tetracycline-dependent tTS^{Kid} repressors, so allowing their reversible repression (and subsequent re-expression) at any stage of breast tumor development. Engineering such mice includes several steps marked as "parallel," which represent "ideal" refinements to the model system that will be undertaken in parallel but are not be absolutely required for preliminary studies on the roles of c-*myc* and *E2f3* target genes in breast cancer.
2. Use HRT-modified mice to determine directly the requirement for c-Myc and/or E2F3 at various stages of tumor development in an H-Ras-driven mouse model of breast cancer and evaluate the therapeutic utility of inhibiting c-Myc and/or E2F3 function in the treatment of breast cancer.
3. Establish the HRT technology platform as a means to assess the requirement for c-Myc and/or E2F3 in any orthotopic mouse model of breast cancer.

Aim 1

Task a. Construct LacI repressor c-Myc knock-in constructs and verify by sequencing (months 1-4).

This has now been achieved. The constructs required sophisticated and painstaking cloning strategies in order to insert the appropriate sequences into the endogenous c-*myc* gene, which delayed completion of all the vectors.

The general strategy for constructing the HRT targeting vector is shown in Figure 1. We deemed it important not to disrupt important *cis*-regulatory elements that might determine the normal pattern of expression of the c-*myc* gene. After substantial groundwork inspecting and assaying the c-*myc* promoter element, the decision was made to insert each regulatory element in the first intron of the c-*myc* gene since, unlike the crowded c-*myc* promoter, this region has no known regulatory elements that might be disrupted by HRT modification.

Construction of the LacI targeting vector required a number of taxing and complex cloning steps, including several three-way blunt ended ligations which, because of their low efficiency, necessitated sorting through large numbers of products to identify correctly ligated vectors. Details of the architecture of the LacI HRT vector are shown in Figure 2. The targeting vector has been assembled and checked by sequencing and restriction fragment digestions.

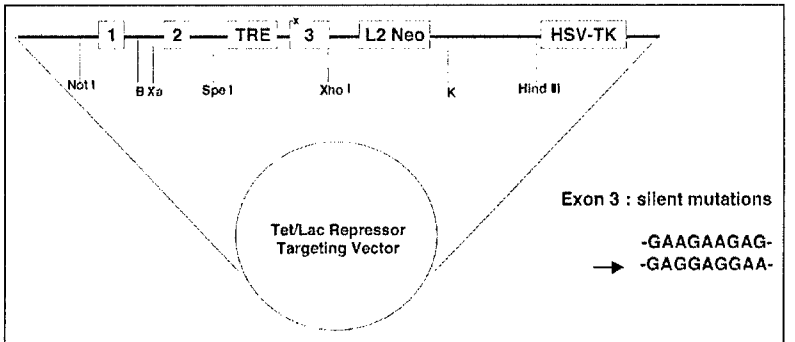

Figure 1 General strategy for modifying endogenous c-*myc* gene

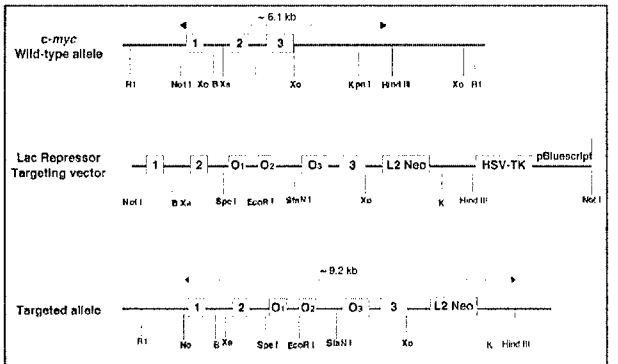

Figure 2 Architecture of LacI HRT targeting vector

Task b. Electroporate constructs into ES cells (transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).

Screening protocols have been successfully developed and tested for identification of correctly targeted ES cells. To facilitate this, a coding-neutral change in the 5' sequence of c-*myc* exon 3 has been introduced into both targeting vectors that will allow for one-step discrimination between targeted and endogenous c-*myc* (marked by the asterisk). Purified DNAs have been electroporated into ES cells and clones tested for targeting.

Task c. Select and expand ES cells and identify ES cells with correctly targeted knock in element (months 5-7).

ES cells with targeted c-*myc* have been identified and cloned. Insertion has been verified by the strategies outlined above.

Task d. Verify IPTG-dependent repression of c-myc expression in ES cells in vitro using TaqMan analysis specific for the modified c-myc transcript (month 8).

Verification is underway and looks very encouraging. Endogenous c-*myc* expression has been tested and appears normal in the absence of IPTG administration.

Task e. Microinject ES cells into blastocysts (UCSF transgenic core) and generate chimeric mice (months 9-11)

Targeted ES cells are currently being microinjected into blastocysts to generate appropriate knock-in chimeric mice.

Task f. *Breed positive chimeric mice to obtain germ line transmission of the modified c-myc gene (months 12-15).*

This task has been will commence once chimeric mice have been transferred to our facility (expected in 6-8 weeks).

Task g. *Start cross with Zp3Cre mice to excise Neo cassette left in the modified c-myc gene (month 14)*

To be completed.

Task h. *Cross LacI-c-Myc KI with beta-actin-(NLS)LacI (months 15-17)*

To be completed. The *beta-actin-(NLS)LacI* have been derived and are now available.

Task i. *Cross HRT LacI-c-cMyc X beta-actin-(NLS)LacI into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with IPTG-switchable c-myc (HRT LacI-c-myc X Wap-H-Ras mice) (months 17-20)*

To be completed.

Task j. *Begin process of backcrossing of chimeras into C57/Black6 background (will need 6 generations, eventually)*

To be completed.

Task k. *Expand colony of HRT LacI-c-myc X Wap-H-Ras mice.*

To be completed.

Task 2

Construct mice in which the endogenous c-*myc* gene is rendered switchable by the Doxycycline-dependent-*tTS*^{Kid} repressor and establish this in the WAP-H-Ras mammary tumor background.

a. *Generate Tet repressor c-Myc knock in construct and verify by sequencing (months 1-4)*

Like the LacI vector, construction of the tTS^{Kid} HRT targeting vector required a number of complex cloning steps, including multiple three-way blunt ended ligations which, again, necessitated sorting through large numbers of products to identify correctly ligated vectors. Details of the architecture of the tTS^{Kid} HRT vector are shown in Figure 3. The targeting vector has been assembled, checked by sequencing and restriction fragment digestions.

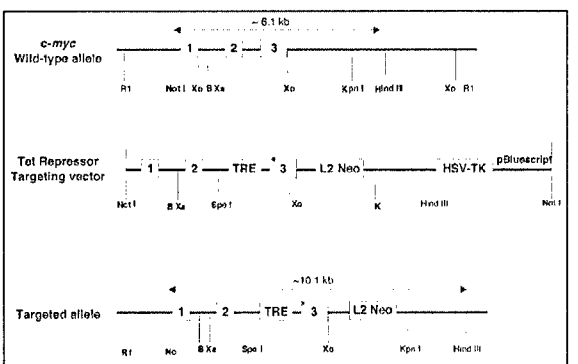

Figure 3 Architecture of tTS^{kid} HRT targeting vector

b. *Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).*

This has been achieved.

c. Select and expand ES cells. Identify ES cells with correctly targeted knock in element (months 5-7).
This has been achieved.

d. Verify doxycycline-dependent repression of c-myc in ES cells in vitro by Taqman expression analysis (month 8).
This is currently in progress but appears to be successful.

e. Microinjection ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11).
Positive ES cell clones are currently being verified prior to micro-injection into blastocycts.

f. Breed positive chimeric mice to obtain germ line transmission of the modified c-myc gene (months 12-15).
To be completed.

g. [parallel] Start cross with Zp3Cre mice to excise Neo cassette left in the modified c-myc gene (month 14).
To be completed.

h. Cross HRT-tet-c-cMyc X beta-actin-tTS^{Kid} mice (months 15-17).
To be completed.

i. Cross HRT-tet-c-cMyc X beta-actin-tTS^{Kid} into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with Tet-switchable c-myc (HRT tTS^{Kid}c-myc X WAP-H-Ras mice) (months 17-20).
To be completed.

j. [parallel] Backcross of chimeras to C57Black6 (6 generations).
To be completed.

k. Expand colony of HRT tTS^{Kid}c-myc X WAP-H-Ras mice.
To be completed.

Task 3

Construct mice in which the endogenous *E2f3* gene is rendered switchable by the IPTG-dependent LacI repressor and establish this in the WAP-H-Ras mammary tumor background.

a. Generate LacI repressor E2F3 knock in construct and verify by sequencing (months 1-4).
The *e2f3* vector required more complex cloning strategies than first planned due to problems with obtaining the *e2f3* genomic sequence from the correct mouse strain. This has now been achieved and vector construction is almost complete.

b. Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).
Execution of this task has been delayed pending successful construction and validation of the *e2f3* targeting vector.

c. Select and expand ES cells and identify ES cells with correctly targeted knock in element (month 5-7).
Execution of this task has been delayed pending successful construction and validation of the *e2f3* targeting vector.

d. Validate IPTG-dependent repression of E2F3 in ES cells in vitro by Taqman expression analysis (month 8).
Execution of this task has been delayed pending successful construction and validation of the *e2f3* targeting vector.

e. Microinject ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11).
Execution of this task has been delayed pending successful construction and validation of the *e2f3* targeting vector.

f. Breed positive chimeric mice to obtain germ line transmission of the modified E2f3 gene (months 12-15).
To be completed.

g. [parallel] Start cross with Zp3Cre mice to excise Neo cassette left in the modified E2f3 gene (month 14).
To be completed.

h. Cross LacI-E2f3 KI with beta-actin-(NLS)LacI (months 15-17).
To be completed.

i. Cross HRT LacI-E2f3 X beta-actin-(NLS)LacI into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with IPTG-switchable E2f3 (HRT LacI-E2f3 X Wap-H-Ras mice) (months 17-20).
To be completed.

j. [parallel] Begin process of backcrossing of chimeras into C57Black6 background (will need 6 generations, eventually).
To be completed.

k. Expand colony of HRT LacI-E2f3 X Wap-H-Ras mice.
To be completed.

Task 4

Determine effect of c-Myc and E2F3-inactivation on normal mouse tissues and on development and maintenance of H-*Ras*-induced breast adenocarcinoma *in vivo*

a. Determine the systemic effects of temporary c-Myc ablation on mouse viability and susceptible tissues (skin, GI tract, bone marrow) after administering IPTG or Dox to, respectively, HRT LacI-c-myc and HRT tTS^{Kid}c-myc mice for varying lengths of time (2 days-2 weeks) (months 20-36).

To be completed.

b. Determine the systemic effects of temporary E2F3 ablation on mouse viability and susceptible tissues (skin, GI tract, bone marrow) after administering IPTG to HRT LacI-E2f3 mice for varying lengths of time (2 days-2 weeks) (months 20-36).

To be completed.

c. Determine effects of short term (as defined in a. and b. above as the longest time that c-Myc or E2F3 can be repressed without significant pathology) on palpable H-Ras-induced breast tumors. Histological and immunohistochemical analysis of tissue samples from adenocarcinomas (months 20-36).

To be completed.

KEY RESEARCH ACCOMPLISHMENTS:

- Successful design, construction and targeting of vector to render endogenous c-*myc* gene regulatable via the IPTG-dependent Lac Repressor. Construction of targeted mouse is proceeding ahead of schedule.

- Successful design, construction and targeting of vector to render endogenous c-*myc* gene regulatable via the tTS^{kid} tetracycline-regulatable. Construction of targeted mouse is on schedule.

- Successful initial stages of targeting endogenous *e2f3* gene for regulation by the IPTG-dependent Lac Repressor. Some unforeseen problems mean that this task is modestly behind schedule, although most recent data are extremely promising.

- Successful establishment of initial colony of *beta-actin-(NLS)LacI* mice on schedule. Animals now inside quarantine barrier at UCSF.

REPORTABLE OUTCOMES:

None as yet.

CONCLUSIONS:

We have successfully implemented much of the first half of our proposed plan. We are on target to able to test our novel model within the next 12 months and evaluate the roles of c-*myc* and *e2f3* function in the genesis, progression and maintenance of H-Ras-induced breast cancer in mice.

REFERENCES:

None